

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
12 December 2002 (12.12.2002)

PCT

(10) International Publication Number
WO 02/099088 A1

- (51) International Patent Classification⁷: **C12N 5/10**, G01N 33/00 (74) Agent: **SCHÜSSLER, Andrea**; Truderinger Strasse 246, 81825 München (DE).
- (21) International Application Number: PCT/EP02/06175 (81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.
- (22) International Filing Date: 5 June 2002 (05.06.2002)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data: 01113805.4 6 June 2001 (06.06.2001) EP (84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).
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Published:

— with international search report

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: DOUBLE-TRANSFECTED CELL LINE USEFUL FOR THE IDENTIFICATION OF TRANSPORT INHIBITORS

(57) Abstract: Described is a double-transfected cell line containing (a) a DNA sequence encoding an uptake transporter for organic anions, preferably OATP8, operatively linked with a promoter and (b) a DNA sequence encoding an export pump for organic anions or anionic conjugates, preferably the multidrug resistance protein 2 (MRP2), operatively linked with a promoter. Moreover, various uses of said cell line are described preferably its use for the identification of transport inhibitors, e.g. drug candidates.



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Double-transfected cell line useful for the identification of transport inhibitors

5 The present invention relates to a double-transfected cell line containing (a) a DNA sequence encoding an uptake transporter for organic anions, preferably OATP8, operatively linked with a promoter and (b) a DNA sequence encoding an export pump for organic anions or anionic conjugates, preferably the multidrug resistance protein 2 (MRP2),
10 operatively linked with a promoter. The present invention also relates to various uses of said cell line, preferably for the identification of transport inhibitors, e.g. drug candidates.

15 Vectorial transport is an important function of all polarized cells contributing to detoxification and to the prevention of entry of toxins into organs. This is exemplified by kidney proximal tubule epithelia, by cells of the blood-brain barrier, by intestinal epithelia, and, last but not least, by hepatocytes. One of the major hepatocellular functions is the
20 removal of endogenous and exogenous substances from the blood circulation and their secretion into the bile. Two transport processes play a decisive role in this vectorial transport by hepatocytes: the sinusoidal (basolateral) uptake from blood and the canalicular (apical) secretion into bile. In human
25 hepatocytes, the sodium-independent uptake of amphiphilic organic anions is mediated by at least three transport proteins, namely by the human organic anion transporters OATP2 (also known as OATP-C or LST1, symbol SLC21A6) (Abe *et al.* 1999; Hsiang *et al.* 1999; König *et al.* 2000a; Cui *et al.* 2001), human OATP8 (SLC21A8) (König *et al.* 2000b; Cui *et al.* 2001), and human OATP-B (SLC21A9) (Kullak-Ublick *et al.* 2001).
30 All three transporters belong to the subgroup 21A of the solute carrier (SLC) superfamily. Whereas OATP-B is expressed

also in a number of other tissues, OATP2 and OATP8 are expressed exclusively in human hepatocytes. The substrate spectrum of OATPs includes bile salts, conjugates of steroid hormones, thyroid hormones, and many other amphiphilic organic anions (Abe et al. 1999; König et al. 2000a, b; Cui et al. 2001; Kullak-Ublick et al. 2000, 2001). Unlike these basolateral uptake transporters which are thought to be of exchanger type (Li et al. 1998), the apical export transporters identified in human hepatocytes so far are members of the ATP-binding cassette (ABC) superfamily (Keppler and Arias 1997; Jansen 2000). The export of organic anions is predominantly mediated by the bile salt export pump BSEP (ABCB11) belonging to the MDR (ABCB) subgroup of the ABC superfamily (Strautnieks et al. 1998; Gerloff et al. 1998; Wang et al. 2001) and by the multidrug resistance protein 2 (MRP2, ABCC2) belonging to the MRP (ABCC) subgroup of the ABC superfamily (Büchler et al. 1996; Suzuki and Sugiyama 1998; König et al. 1999; Borst et al. 2000). While the major substrates of BSEP are bile salts like cholestyramine and cholate (Gerloff et al. 1998), the organic anions transported by MRP2 are mainly conjugates of lipophilic substances with glutathione, glucuronate, or sulfate (Evers et al. 1998; Cui et al. 1999, König et al. 1999).

The transhepatic transport of amphiphilic organic anions has been frequently studied by use of model compounds like sulfobromophthalein (BSP) and indocyanine green (ICG) (Scharschmidt et al. 1975). Functional characterization of the three human OATPs identified in the hepatocyte basolateral membrane demonstrated that all three are able to mediate the uptake of BSP, with the highest affinity for OATP2 ($K_m = 140$ nM) and the lowest affinity ($K_m = 3.4 \mu M$) for OATP8 (König et al. 2000b; Kullak-Ublick et al. 2001; Cui et al. 2001). In the hepatocyte BSP is predominantly conjugated with glutathione to yield the BSP glutathione S-conjugate (BSP-SG) (Whelan et al. 1970; Snel et al. 1995). Studies with transport-deficient mutant rats (Jansen et al. 1987) which lack the canalicular export pump Mrp2 (Paulusma et al. 1996; Büchler et al. 1996;

Ito et al. 1997) suggested that this export pump mediates the secretion of BSP-SG into bile. However, it was not established that BSP itself is a substrate for human MRP2. So far, the transport proteins like OATPs and MRPs were studied mostly by use of transfected mammalian cells or by use of *Xenopus* oocyte systems expressing only one exogenous recombinant transport protein (Madon et al. 1997; Ito et al. 1998; Evers et al. 1998; Cui et al. 1999, 2001; Abe et al. 1999; Hsiang et al. 1999; König et al. 2000a, b; Kullak-Ublick et al. 2001).

When developing or designing new pharmaceuticals one of the critical questions is to whether the candidate compounds have undesired side effects like interference with the transport of substances which are produced or occur naturally in the body, e.g. interference with the hepatic or renal transport of organic anions, as exemplified by the interference of rifampicin, rifamycin SV, or CDNB with the transcellular transport of BSP. For cost saving, it is desirable to detect such side effects of drug candidates at an early stage of development. So far, for the study of potential side effects of drug candidates animals or cell cultures were used. However these approaches exhibit a variety of disadvantages, e.g. are time and cost consuming, do not allow the high throughput screening of drug candidates etc.

Therefore, it is the object of the present invention to provide a means for the efficient analysis of the interference of a drug candidate with transport of substances which are produced or occur naturally in the body, particularly interference with the hepatic or renal transport of organic anions which overcomes the disadvantages of the systems presently used.

According to the invention this is achieved by the subject matters defined in the claims. A cell system with defined human uptake and export transporters was established, i.e. a double-transfected MDCK cell line permanently expressing a recombinant uptake transporter for organic anions in the

basolateral membrane and an ATP-dependent export pump for anionic conjugates in the apical membrane. Basolateral uptake was mediated by the human organic anion transporter OATP8 (symbol SLC21A8) and subsequent apical export by the multidrug resistance protein 2 (MRP2; symbol ABCC2). Under physiological conditions, both transport proteins are strongly expressed in hepatocytes and contribute to the hepatobiliary elimination of organic anions. Expression and localization of OATP8 and MRP2 in MDCK cells growing on Transwell membrane inserts was demonstrated by immunoblotting and confocal laser scanning microscopy. ³H-Labeled sulfobromophthalein (BSP) was a substrate for both transport proteins and was transferred from the basolateral to the apical compartment at a rate at least 6 times faster by double-transfected MDCK-MRP2/OATP8 cells than by single-transfected MDCK-OATP8 or MDCK-MRP2 cells. Vectorial transport at a much higher rate by double-transfected than by single-transfected cells was also observed for the ³H-labeled substrates leukotriene C₄, 17 β -glucuronosyl estradiol, and dehydroepiandrosterone sulfate, for the fluorescent anionic substrate fluo-3, and for the antibiotic rifampicin. Inhibition studies indicated that intracellular formation of S-(2,4-dinitrophenyl)-glutathione from 2,4-chlorodinitrobenzene selectively inhibits the transcellular transport of [³H]BSP at the site of MRP2-mediated export.

The identification of the new substrates for MRP2 and OATP8 demonstrates that the double-transfected cell line is useful for the characterization of these transporters. In comparison to MDCK cells transfected separately with either OATP8 or MRP2, the double-transfectants have several advantages. It was so far difficult to study MRP2 function in whole cells because most substrates for MRP2 are negatively charged under physiological conditions and thus can not penetrate the plasma membrane without an uptake transporter. Therefore, MRP2 has been mostly studied using inside-out membrane vesicles prepared from MRP2-expressing cells. With the double-transfected MDCK cells expressing OATP8 and MRP2 and with compounds like [³H]BSP and Fluo-3, which are substrates for

both transporters, we may now screen more easily for MRP2 inhibitors with intact cells. An inhibitor for only MRP2 will inhibit the transcellular transport and enhance the intracellular accumulation of [³H]BSP. An inhibitor for both MRP2 and OATP8 will reduce the transcellular transport more strongly than the intracellular accumulation of [³H]BSP. Because of the easier handling of double-transfected cells grown on Transwell membrane inserts in comparison with the preparation and handling of membrane vesicles, it is possible to develop high-throughput screening systems for MRP2 inhibitors by the use of the double-transfected cells. The use of the fluorescent penta-anion Fluo-3 as a substrate for both OATP8 and MRP2 may further facilitate the screening.

To conclude, the double-transfected cells of the present invention provide a useful system for the identification of transport substrates and transport inhibitors including drug candidates. By using the double-transfected cell line of the present invention the inhibiting effect of drug candidates on defined transport proteins which are, e.g., present in liver, kidney, intestine or blood brain barrier can be investigated.

Accordingly, the present invention provides a double-transfected cell line, preferably a stable cell line containing (a) a DNA sequence encoding an uptake transporter for organic anions operatively linked with a promoter and (b) a DNA sequence encoding an export pump for organic anions or anionic conjugates operatively linked with a promoter. Preferably, the uptake transporter for organic anions is expressed in the basolateral cell membrane and the export pump for organic anions or anionic conjugates is expressed in the apical cell membrane.

Preferably, for transfecting the cells the DNA sequences are present in a vector or expression vector. A person skilled in the art is familiar with examples thereof. The DNA sequences can also be contained in a recombinant virus containing appropriate expression cassettes. Suitable viruses that may be

used in the present invention include baculovirus, vaccinia, sindbis virus, SV40, Sendai virus, adenovirus, an AAV virus or a parvovirus, such as MVM or H-1. The vector may also be a retrovirus, such as MoMULV, MoMuLV, HaMuSV, MuMTV, RSV or GaLV. For expression in mammals, a preferred suitable promoter is human cytomegalovirus "immediate early promoter" (pCMV).

For generating the above described DNA sequences and for constructing expression vectors which contain said DNA sequences, it is possible to use general methods known in the art. These methods include e.g. in vitro recombination techniques, synthetic methods and in vivo recombination methods as described in Sambrook et al., Molecular Cloning, A Laboratory Manual, 2nd edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, for example. Methods of transfecting the cells, of phenotypically selecting transfectants and of expressing the DNA according to the invention by using the above described vectors are known in the art.

Preferably, the cell line is a human cell line, e.g. HEK293. Particularly preferred are polarized cells, e.g. hepatocytes, kidney cells, e.g. MDCKII or HepG2.

In a further preferred embodiment of the double-transfected cell line of the present invention the uptake transporter for organic anions is a member of the subgroup 21A of the solute carrier (SLC) superfamily. Particularly preferred are OAT1 (SLC22A6) (Hosoyamada et al. 1999), OATP2, OATP8 or OATP-B.

In a more preferred embodiment of the double-transfected cell line of the present invention the export pump for organic anions or anionic conjugates is a member of the MDR (ABCB) subgroup or the MRP (ABCC) subgroup of the ABC superfamily. Particularly preferred are the bile salt export pump BSEP (ABCB11) or the multidrug resistance protein 2 (MRP2). A combination of OAT1 with MRP2, both of which are expressed in human kidney proximal tubule cells (Tojo et al. 1999; Schaub

et al. 1999), may serve to study the renal clearance of organic anions.

In an even more preferred of the double-transfected cell line of the present invention the DNA sequence encoding an uptake transporter for organic anions and/or the DNA sequence encoding an export pump for organic anions or anionic conjugates are operatively linked with a promoter allowing high expression.

In certain cases for investigating the effect of the intracellular drug metabolism on transport it might be desirable to transfect the cells of the invention with a DNA sequence encoding a third compound the nature of which may vary according to the drug to be studied.

Finally, the present invention relates to various uses of the double-transfected cell line. A preferred use is the identification of a transport substrate or a transport inhibitor, particularly a drug candidate. Suitable assays formats are known to the person skilled and, e.g., described in the examples, below. A preferred assay format is high throughput screening.

Brief description of the drawings

Fig. 1. Immunoblot analysis of MRP2 and OATP8 in transfected MDCK cells. Crude membrane fractions from MDCK cells permanently transfected with control vector (MDCK-Co), with human *MRP2* (MDCK-MRP2), with human *OATP8* (MDCK-OATP8), or with both *MRP2* and *OATP8* cDNA (MDCK-MRP2/OATP8) were separated by SDS-PAGE. A, Human MRP2 was detected by the polyclonal antibody EAG5 (Keppler and Kartenbeck 1996; Schaub *et al.* 1999). B, Human OATP8 was detected by the polyclonal antibody SKT (König *et al.* 2000b). In case of OATP8, only the fully glycosylated form is indicated by an arrowhead, whereas the band at about 90 kDa

represents an under-glycosylated form of the protein (König et al. 2000b).

Fig. 2. Immunolocalization of recombinant MRP2 and OATP8 in MDCK cells. MDCK cells expressing OATP8 alone (A, D) or both MRP2 and OATP8 (B, C, E, and F) were grown on Transwell membrane inserts and examined by confocal laser scanning microscopy. OATP8 (green fluorescence) and MRP2 (red fluorescence) were stained using the polyclonal antibody SKT (König et al. 2000b) and the monoclonal antibody M₂III6 (Evers et al. 1998), respectively. A and B are en face images focused at the middle of the cell monolayer, C is an en face image focused at the top of the cell monolayer. D, E, and F are vertical sections at the positions indicated by the white lines in A, B, and C. Besides the lateral localization of OATP8, some intracellular staining of this protein can be seen in the vertical sections. Only MRP2 is localized to the apical membrane (E, F). Bar, 10 μ m.

Fig. 3. Scheme of the MDCK transfectants growing on the Transwell membrane inserts as a polarized monolayer. The apical membrane facing the culture medium is separated from the basolateral membrane facing the membrane support by the tight junctions. The export pump MRP2 is localized to the apical membrane, whereas the uptake transporter OATP8 is localized to the basolateral membrane.

Fig. 4. Transcellular transport of [³H]BSP. MDCK-Co (ê), MDCK-MRP2 (s), MDCK-OATP8 (c), and MDCK-MRP2/OATP8 (g) cells were grown on Transwell membrane inserts. [³H]BSP (1 μ M) was given to the basolateral compartments. At the time points indicated, radioactivity in the apical compartments (Transcellular [³H]BSP transport) and inside the cells (Intracellular [³H]BSP accumulation) was

determined. Total uptake of [3 H]BSP was calculated as the sum of intracellular and apical radioactivity. Data represent means \pm SD ($n = 4$). For most measurements the standard deviation was within the size of the symbols.

Fig. 5. Vectorial transport and efflux of [3 H]BSP. MDCK-OATP8 and MDCK-MRP2/OATP8 cells were grown on Transwell membrane inserts. A, Vectorial transport of [3 H]BSP. [3 H]BSP ($1 \mu\text{M}$) was given either to the basolateral compartments (B @ A) or to the apical compartments (A @ B). After 15 min at 37°C , radioactivity in the opposite compartments was measured. B, Efflux of [3 H]BSP. MDCK-OATP8 and MDCK-MRP2/OATP8 cells were incubated with [3 H]BSP ($1 \mu\text{M}$) in the basolateral compartments at 37°C for 30 min. The cells were then washed with cold buffer and incubated with buffer without [3 H]BSP at 37°C for 30 min. The radioactivity subsequently released into the basolateral and the apical compartment and inside the cells was measured. Data represent means \pm SD ($n = 4$).

Fig. 6. HPLC analyses of [3 H]BSP. MDCK-MRP2/OATP8 cells grown on Transwell membrane inserts were incubated with $1 \mu\text{M}$ [3 H]BSP in the basolateral compartment at 37°C for 30 min. The medium in the apical compartment and the cell lysate were analyzed by radio-HPLC as described in "Materials and Methods". A, Authentic [3 H]BSP (Cui et al. 2001); B, radioactivity collected in the apical compartment; C, radioactivity accumulated in the cells. [3 H]BSP (arrow head) and the glutathione S-conjugate of [3 H]BSP ([3 H]BSP-SG, arrow) are indicated. Acivicin, an inhibitor of the degradation of the glutathione moiety of [3 H]BSP-SG, was added to the incubation at a concentration of 5 mM.

Fig. 7. ATP-dependent transport of [3 H]BSP by human MRP2. A, Inside-out membrane vesicles from HEK293 cells transfected with human MRP2 (HEK-MRP2) were incubated with 1 μ M [3 H]BSP in the presence of ATP (g) or 5'-AMP (c). B, Net ATP-dependent transport of [3 H]BSP into the vesicles from HEK-MRP2 cells (g) or HEK-Co cells (c) was calculated by subtracting the values determined in the presence of 5'-AMP from those in the presence of ATP. C, The K_m value of human MRP2 for BSP was determined at BSP concentrations between 1 and 10 μ M. Data represent means \pm SD (n = 4).

Fig. 8. Transcellular transport of organic anions. MDCK-Co, MDCK-OATP8, and MDCK-MRP2/OATP8 cells grown on Transwell membrane inserts were incubated with [3 H]BSP (1 μ M), [3 H]LTC₄ (0.5 μ M), [3 H]E₂17bG (5 μ M), [3 H]DHEAS (5 μ M), Fluo-3 (2 μ M) or [3 H]cholyt taurine (5 μ M) in the basolateral compartments at 37°C. The radioactivity (labeled substrates) or fluorescence (Fluo-3) in the apical compartments was then measured after 30 min. Data represent means \pm SD (n = 4).

Fig. 9. Inhibition of the transcellular transport of [3 H]BSP. MDCK-OATP8 (A, C, E, G), and MDCK-MRP2/OATP8 (B, D, F, H) cells grown on Transwell membrane inserts were incubated with [3 H]BSP (1 μ M) in the basolateral compartments in the presence of different concentrations of human serum albumin (HSA, C, D), rifampicin (E, F), or rifamycin SV (G, H). For 2,4-chloro-dinitrobenzene (CDNB) (A, B), cells were pre-incubated with CDBN at room temperature for 20 min, transport of [3 H]BSP was started by replacing the buffer in the basolateral compartments by fresh buffer containing [3 H]BSP and CDBN. After incubation for 30 min at 37°C, the

radioactivity in the apical compartments and inside the cells was measured. Data represent means \pm SD (n = 4).

Fig.10. Transcellular transport of rifampicin. MDCK-Co, MDCK-MRP2, MDCK-OATP8, and MDCK-MRP2/OATP8 cells grown on Transwell membrane inserts were incubated with 50 μ M rifampicin in the basolateral compartments at 37°C for 30 min. The concentration of rifampicin in the apical compartments was determined by the specific absorption of rifampicin at 475 nm. Data represent means \pm SD (n = 4).

Fig. 11: Immunolocalisation of recombinant transport proteins in double-transfected cell lines. MDCKII cells transfected with OAT1 and MRP2 (A,C) or with OATP2 and MRP2 (B,D) were grown on Transwell membrane inserts. Recombinant MRP2 was stained with the antiserum EAG5 (green in A,C) or with the commercial antibody M₂III6 (red in B, D). Recombinant OAT1 was stained with a commercial monoclonal antibody against an epitope at the carboxyl terminus of OAT1 (red in A, C). Recombinant OATP2 was stained with the polyclonal antiserum ESL (green in B, D). In both double-transfected cell lines, MRP2 was localized to the apical membrane of polarized MDCKII cells. OAT1 and OATP2 were detected only in the basolateral membrane of the respective cell lines.

Fig. 12: Transcellular transport of para-aminohippurate (PAH) by MDCK-OAT1/MRP2 cells. MDCKII cells transfected with control vector (MDCK-Co), with OAT1 (MDCK-OAT1), or with both OAT1 and MRP2 (MDCK-OAT1/MRP2) were grown on Transwell membrane inserts and incubated with 10 μ M [³H]PAH added to the basolateral compartment at 37°C. Radioactivity accumulated in the apical compartment was measured after 30 min.

Fig. 13: Transcellular transport of BSP by MDCK-OATP2/MRP2 cells. MDCKII cells transfected with control vector (MDCK-Co), with OATP2 (MDCK-OATP2), or with both OATP2 and MRP2 (MDCK-OATP2/MRP2) were grown on Transwell membrane inserts and incubated with 1 μ M [³H]BSP added to the basolateral compartment at 37°C. Radioactivity accumulated in the apical compartment was measured after 30 min.

Abbreviations: ABC, ATP-binding cassette superfamily; BSEP, bile salt export pump; BSP, sulfobromophthalein; CDNB, 1-chloro-2,4-nitrobenzene, DHEAS, dehydroepiandrosterone 3-sulfate; DiOC6(3), 3,3'-dihexyloxacarbocyanine iodide; E₂17bG, 17b-glucuronosyl estradiol; Fluo-3, 1-[2-amino-5-(2,7-dichloro-6-hydroxy-3-oxo-3H-xanthen-9-yl)]-2-(2'-amino-5'-methylphenoxy)-ethane-N,N,N',N'-tetraacetic acid penta-ammonium salt; HEK293, human embryonic kidney cells; HPLC, high-performance liquid chromatography; HSA, human serum albumin; K_m, Michaelis-Menten constant; LTC₄, leukotriene C₄; MDCKII, Madin-Darby canine kidney cells, strain II; MRP2, multidrug resistance protein 2; OAT1, organic anion transporter 1; OATP, human organic anion-transporting polypeptide; OCT1, organic cation transporter 1; SLC, solute carrier superfamily; TC, taurocholate or cholyl taurine; SDS, sodium dodecyl sulfate

The present invention is explained by the examples.

Example 1: Materials and Methods

(A) **Chemicals.** [^3H]BSP (0.5 TBq / mmol) was obtained from Hartmann Analytic (Köln, Germany) by custom synthesis (Cui et al. 2001). [$^{14,15,19,20-3}\text{H}$]LTC₄, [$^{1,2,6,7-3}\text{H}$]dehydroepiandrosterone sulfate (0.6 TBq / mmol), [^3H]cholylyl taurine (73 GBq / mmol), and 17b-D-glucuronosyl [$^{6,7-3}\text{H}$]estradiol (1.6 TBq / mmol) were purchased from Perkin-Elmer Life Science Products (Boston, MA). [^{14}C]Inulin carboxylic acid (82 MBq / g) was obtained from Biotrend Chemicals (Köln, Germany). Fluo-3 (1-[2-amino-5-(2,7-dichloro-6-hydroxy-3-oxo-3H-xanthen-9-yl)]-2-(2'-amino-5'-methyl-phenoxy)-ethane-N,N,N',N'-tetraacetic acid penta-ammonium salt) was from Calbiochem (Bad Soden, Germany). Rifampicin, rifamycin SV, acivicin, and CDNB were purchased from Sigma (Deisenhofen, Germany). G418 (geneticin) sulfate was from Life Technologies (Gaithersburg, MD). Hygromycin was from Invitrogen (Groningen, Netherlands). Additional non-radioactive chemicals of analytical purity were obtained from Sigma.

(B) **Cell Culture and Transfection.** HEK293 (human embryonic kidney) and MDCKII (strain II of Madin-Darby canine kidney) cells were cultured in minimum essential medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin at 37°C, 95% humidity, and 5% CO₂. HEK-MRP2 and HEK-Co are HEK293 cells transfected with human *MRP2* cDNA and control vector, respectively; MDCK-MRP2 and MDCK-Co are MDCKII cells transfected with human *MRP2* cDNA and control vector, respectively, as described (Cui et al. 1999).

The human *OATP8* cDNA (König et al. 2000b) was subcloned into the mammalian expression vector pcDNA3.1/Hygro(+) (Invitrogen) and transfected into MDCKII cells using the polybrene method (König et al. 2000b). Transfectants expressing recombinant *OATP8* were selected with

hygromycin (950 μM). The clone with the highest OATP8 expression (MDCK-OATP8) was further transfected with the vector construct pcDNA3.1-MRP2 with the full-length human MRP2 cDNA (Cui et al. 1999). After selection with 950 μM hygromycin and 800 μM G418 disulfate for three weeks, the transfectants were screened for both MRP2 and OATP8 expression by immunoblot analyses. A clone with the highest MRP2 expression and a similar expression level of OATP8 as MDCK-OATP8 cells was designated as MDCK-MRP2/OATP8 and chosen for further studies.

(C) Immunoblot Analysis. Crude membrane fractions were prepared from cultured MDCKII cells as described earlier (Cui et al. 1999). Proteins were separated by SDS-PAGE (7.5% gels). OATP8 was detected by the polyclonal antibody SKT (König et al. 2000b) at a dilution of 1:5000 in TBS-T (20 mM Tris, 145 mM NaCl, 0.05% Tween 20, pH 7.6). MRP2 was detected by the polyclonal antibody EAG5 (Büchler et al., 1996; Keppler and Kartenbeck, 1996) at a dilution of 1:10000 in TBS-T.

(D) Confocal Laser Scanning Immunofluorescence Microscopy. MDCKII cells were grown on Transwell membrane inserts (6.5 mm diameter, 0.4 μm pore size, Corning Costar, Bodenheim, Germany) for 3 days at confluency and induced with 10 mM sodium butyrate for 24 h (Cui et al. 1999). Cells were fixed with 2.5% paraformaldehyde in PBS (137 mM NaCl, 2.7 mM KCl, 8.0 mM Na_2HPO_4 , 1.5 mM KH_2PO_4 , pH 7.4) for 20 min, permeabilized with 1% Triton X-100 in PBS for 20 min, and incubated for 1.5 h with primary antibodies at room temperature. The polyclonal antibody SKT (König et al. 2000b), at a dilution of 1:25 in PBS, and the monoclonal mouse antibody M₂III-6 (Alexis Biochemicals, Grünberg, Germany) at a dilution of 1:20 in PBS, were used to stain OATP8 and MRP2, respectively. Cells were then washed three times with PBS and incubated with secondary antibodies. Both Cy2-conjugated goat anti-rabbit IgG and Cy3-conjugated goat anti-mouse IgG were

obtained from Jackson Laboratories (West Grove, PA) and used at a dilution of 1:200 in PBS. The Transwell membranes were then cut from the membrane inserts and mounted using 50% glycerol in PBS onto the slides. Confocal laser scanning microscopy was performed with a LSM 510 apparatus from Zeiss (Oberkochen, Germany).

(E) Transport Assays. For transport assays MDCKII cells were grown on Transwell membrane inserts (24 mm diameter, 0.4 μ m pore size, Corning Costar) at confluency for 3 days and induced with 10 mM sodium butyrate for 24 h. Cells were first washed with transport buffer (142 mM NaCl, 5 mM KCl, 1 mM KH_2PO_4 , 1.2 mM MgSO_4 , 1.5 mM CaCl_2 , 5 mM glucose, and 12.5 mM HEPES, pH 7.3), subsequently, ^3H -labeled substrates were added in transport buffer either to the apical compartments (1 ml) or to the basolateral compartments (1.5 ml). After the time periods indicated, the radioactivity in the opposite compartments was measured. The intracellular accumulation of the radioactivity was determined by lysing the cells with 2 ml 0.2% SDS in water and measuring the radioactivity in cell lysates.

To study the transcellular transport of Fluo-3, cells were incubated with 2 μ M Fluo-3 in the basolateral compartments at 37°C for 30 min. The fluorescence of Fluo-3 in the apical compartment was measured with a RF-510 fluorescence spectrometer (Shimadzu, Duisburg, Germany) at an excitation wave length of 506 nm (5 nm bandwidth) and an emission wavelength of 526 nm (10 nm bandwidth) in the presence of 1.5 mM Ca^{2+} (Nies et al. 1998).

To study the transcellular transport of rifampicin, cells were incubated with 50 μ M rifampicin in the basolateral compartments at 37°C for 30 min. To determine the concentration of rifampicin in the apical compartments its absorption at 475 nm was measured with a spectrophotometer (Ultrospec III, Amersham-Pharmacia, Freiburg, Germany). A calibration curve

for the calculation of the concentrations of rifampicin was determined in the concentration range between 1 and 50 μM .

5 For inhibition studies the inhibitors were added simultaneously with [^3H]BSP into the basolateral compartments, with the exception of 1-chloro-2,4-dinitrobenzene (CDNB). In this case, MDCKII cells grown on Transwell membrane inserts were pre-incubated with CDNB in transport puffer in both apical and basolateral compartments for 20 min at room
10 temperature. The transcellular transport was then started by replacing the buffer in the basolateral compartments by fresh buffer containing CDNB and [^3H]BSP.

15 The transcellular leakage was determined by incubating cells with 50 μM [^{14}C]inulin carboxylic acid in the basolateral compartments for 30 min and measuring the radioactivity in the apical compartments. For all four MDCKII cell lines used in this work the transcellular leakage was lower than 1%.

20 **HPLC Analysis of [^3H]BSP.** MDCK-MRP2/OATP8 cells grown on Transwell membrane inserts were incubated with 1 μM [^3H]BSP in the basolateral compartment for 30 min at 37°C after a pre-incubation with 5 mM acivicin, an inhibitor of g-glutamyltransferase (Allen et al. 1980), in both compartments
25 for 30 min at 37°C. The radioactivity in the apical compartment and in the cell lysate was analyzed by HPLC. Reversed-phase HPLC analyses on a C₁₈ Hypersil column (5-mm particles; Shandon, Runcorn, UK) were performed as described (Cui et al. 2001) using a linear gradient elution from 100% buffer A (45% methanol / 55% water containing 2 mM tetrabutylammonium
30 hydroxide at pH 6.0) to 100% buffer B (90% methanol / 10% water containing 2 mM tetrabutylammonium hydroxide at pH 6.0) at a flow rate of 1 ml/min. [^3H]BSP-SG was synthesized for the HPLC analyses by incubating 1 mM GSH, 0.4 mM [^3H]BSP (1 μCi),
35 and 5 mM acivicin with 350 μl mouse liver cytosol in a final volume of 500 μl for 1 h at 37°C. After deproteinization with 3 volume of methanol 200 μl supernatant was analyzed by HPLC

as described above.

Vesicle Transport Studies. Transport of [³H]BSP into membrane vesicles was measured by the rapid filtration method (Keppler et al. 1998). Briefly, membrane vesicles (30 µg protein) were incubated in the presence of 4 mM ATP, 10 mM creatine phosphate, 100 µg/ml creatine kinase, and [³H]BSP in an incubation buffer (250 mM sucrose, 10 mM Tris/HCl, pH 7.4) in a final volume of 55 µl at 37°C. Aliquots (15 µl) were taken at the indicated time points, diluted in 1 ml ice-cold incubation buffer, and immediately filtered through presoaked nitrocellulose membrane (0.2 µm pore size, Schleicher & Schüll, Dassel, Germany). Filters were rinsed twice with 5 ml of incubation buffer, dissolved in liquid scintillation fluid, and counted for radioactivity. In control experiments, ATP was replaced by an equal concentration of 5'-AMP. ATP-dependent transport was calculated by subtracting values obtained in the presence of 5'-AMP from those obtained in the presence of ATP.

Example 2: Expression and Localization of Recombinant Human OATP8 and MRP2 in MDCKII cells

The expression of human OATP8 and MRP2 in the transfected MDCKII cells was first analyzed by immunoblotting (Fig. 1). As shown in Fig. 1A, MRP2 expression was detected by the antibody EAG5 in MDCKII cells transfected with *MRP2* cDNA alone (MDCK-MRP2) or with both *OATP8* and *MRP2* cDNA (MDCK-MRP2/OATP8). The expression of OATP8 was detected by the antibody SKT in MDCKII cells transfected with *OATP8* cDNA alone (MDCK-OATP8) and in MDCK-MRP2/OATP8 cells (Fig. 1B). Consistent with our earlier report (König et al. 2000b), the fully glycosylated form of human OATP8 has an apparent molecular weight of about 120 kDa, the bands with lower apparent molecular weight detected by the antibody SKT resulted from the under-glycosylated forms of OATP8. In the MDCKII cells transfected with the control vector (MDCK-Co) neither expression of human OATP8 nor MRP2 could be detected (Fig. 1).

The cellular localization of the recombinant transporters in the transfectants was studied by means of immunofluorescence and confocal laser scanning microscopy. In MDCK-OATP8 cells, OATP8 could be stained with the antibody SKT in the basolateral membrane (Fig. 2A and D). In these cells, also some intracellular staining of OATP8 was observed. Double-labeling experiments using the antibody SKT and DiOC₆(3) which stains specifically the endoplasmic reticulum (Terasaki *et al.* 1984) indicated that the intracellular OATP8 fraction was localized to the ER (not shown). The partial ER localization of OATP8 corresponds to its biogenesis and is consistent with the existence of under-glycosylated OATP8 observed in immunoblot analyses (Fig. 1B). In MDCK-MRP2/OATP8 cells, MRP2 was localized to the apical membrane, in addition to the lateral appearance of OATP8 (Fig. 2B, C, E, and F). Fig. 2B shows an image focused in the plane of the nuclei where only OATP8 was visible in this plane. Fig. 2C shows an image focused at the top of the cells, where the MRP2 staining could be seen. In vertical sections both OATP8 and MRP2 were visible (Fig. 2E and 2F). Fig. 3 shows schematically the role of both recombinant transporters in the vectorial transport by the respective transfected cell line.

Example 3: Transcellular Transport of [³H]BSP Mediated by OATP8 and MRP2

The function of human OATP8 and MRP2 in the double-transfected cells was studied by measurement of the transcellular transport of the organic anion [³H]BSP, a substrate of human OATP8 (König *et al.* 2000b, Cui *et al.*, 2001). Thus, polarized MDCKII cells grown on Transwell membrane inserts were incubated with [³H]BSP at a concentration of 1 μ M in the basolateral compartments. At different time points, the radioactivity accumulated in the apical compartment and in the cells was measured. As shown in Fig. 4A, the intracellular accumulation of [³H]BSP was significantly higher in MDCKII cells transfected either with OATP8 cDNA alone (MDCK-OATP8) or

with both *OATP8* and *MRP2* cDNA (MDCK-MRP2/*OATP8*) than in the control-transfected (MDCK-Co) and in the *MRP2*-transfected (MDCK-MRP2) cells, demonstrating that *OATP8* is necessary for the intracellular accumulation of [³H]BSP. However, when the radioactivity in the apical compartment was measured, no significant transfer of [³H]BSP from the basolateral compartments into the apical compartments could be observed for MDCK-Co, MDCK-MRP2, and MDCK-*OATP8* cells (Fig. 4B), whereas a marked transcellular transport of [³H]BSP could be observed with MDCK-MRP2/*OATP8* cells (Fig. 4B). Fig. 4C demonstrates a higher total uptake of [³H]BSP (intracellular accumulation plus transcellular transport) by the MDCK-MRP2/*OATP8* cells than by the MDCK-*OATP8* cells.

The transcellular transport of [³H]BSP by the MDCK-MRP2/*OATP8* cells was a vectorial process, as shown in Fig. 5A. Only a basolateral to apical transport of [³H]BSP was observed, whereas the apical to basolateral transport was negligible. This was expected from the cellular localization of *OATP8* and *MRP2* in the double-transfectants and from the unidirectionality of *MRP2*-mediated transport (Fig. 3).

The efflux of [³H]BSP was determined after incubation of MDCK-*OATP8* and MDCK-MRP2/*OATP8* cells with 1 μ M [³H]BSP for 30 min in the basolateral compartment. As shown in Fig. 5B, [³H]BSP was released from the MDCK-*OATP8* cells mainly across the basolateral membrane, whereas it was exported from the MDCK-MRP2/*OATP8* cells mainly across the apical membrane. When the radioactivity remaining in the cells after the 30 min efflux experiments was compared, the MDCK-MRP2/*OATP8* cells showed a significantly lower intracellular [³H]BSP level than the MDCK-*OATP8* cells, indicating the efficient export via *MRP2* in the apical membrane.

Example 4: BSP Itself and not its Glutathione S-Conjugate is Transported by MRP2 in the Double-transfected MDCK Cells

5 Studies in rat liver showed that BSP is secreted into bile mainly as glutathione conjugate (Combs 1965; Klaassen and Plaa 1967). To investigate whether this is also true for the double-transfectants, we checked the radioactivity in the apical compartment of the MDCK-MRP2/OATP8 cells by radio-HPLC
10 after incubation of the cells with 1 μ M [3 H]BSP in the basolateral compartment for 30 min. The majority of the radioactivity (> 98%) that was accumulated in the apical compartment (Fig. 6B) and in the cells (Fig. 6C) showed the same retention time (17 min) as the unconjugated [3 H]BSP
15 (Fig. 6A). Only a small peak of [3 H]BSP-SG (retention time 15 min) was observed in the apical compartment (Fig. 6B). These results suggest that BSP is taken up by human OATP8 and is itself, rather than its glutathione S-conjugate, a substrate for MRP2 during transcellular transport. To confirm this
20 hypothesis we investigated the transport of [3 H]BSP into inside-out membrane vesicles prepared from HEK-MRP2 cells (HEK293 cells transfected with human MRP2, Cui et al. 1999). As shown in Fig. 7A, [3 H]BSP was transported ATP-dependently into membrane vesicles from HEK-MRP2 cells. The accumulation
25 of [3 H]BSP in the membrane vesicles from HEK-MRP2 cells was significantly higher than in the membrane vesicles from control-transfected HEK-Co cells (Fig. 7B) demonstrating that [3 H]BSP is a substrate for human MRP2. A K_m value of 12 μ M for BSP was determined for MRP2 (Fig. 7C).

Example 5: Transcellular Transport of Other Organic Anions

35 Transcellular transport of other organic anions which are substrates for both OATP8 and MRP2 was also studied. [3 H]Leukotriene C₄ (LTC₄), 17 β -glucuronosyl [3 H]estradiol (E₂17 β G), and [3 H]dehydroepiandrosterone sulfate (DHEAS) have

already been identified as OATP8 substrates (König *et al.* 2000b; Kullak-Ublick *et al.* 2001; Cui *et al.* 2001). [³H]LTC₄ and [³H]E₂17bG have been shown to be high affinity substrates for MRP2 (Cui *et al.* 1999). Our studies also identified
5 [³H]DHEAS as a substrate of MRP2 (Gologan, Leier, and Keppler, unpublished data, 2001). Similar to [³H]BSP (Fig. 8A), all three compounds were transported with much higher velocities across MDCK-MRP2/OATP8 cells than across MDCK-OATP8 or MDCK-Co cells (Fig. 8B, C, D). Fluo-3 is a fluorescent compound (Minta
10 *et al.* 1989) and a good substrate for MRP2 (Nies *et al.* 1998). Like the other compounds mentioned above, Fluo-3 was transported across the MDCK-MRP2/OATP8 cell monolayer with a higher transport rate in comparison to the MDCK-OATP8 cell monolayer (Fig. 8E), suggesting that Fluo-3 is also a
15 substrate for human OATP8.

In Fig. 8F the transcellular transport of [³H]cholyl taurine (TC), which is neither a substrate for human OATP8 (König *et al.* 2000b, Cui *et al.* 2001) nor for MRP2 (Madon *et al.* 1997),
20 was studied. Unlike the other compounds tested, [³H]cholyl taurine was transported across all three MDCKII cell lines with a quite high velocity, but no difference in transport rates was observed between MDCK-Co, MDCK-OATP8, and MDCK-MRP2/OATP8 cells. The high transport rate for [³H]cholyl
25 taurine by the MDCK cells is probably a result of the expression of endogenous transport systems for bile salts.

Example 6: Inhibition of Transcellular [³H]BSP Transport

30 The hydrophobic compound CDNB which is thought to enter cells via diffusion is conjugated with glutathione inside the cell and then pumped out via MRP2 (Evers *et al.* 1998). These properties of CDNB allowed us to differentiate between the
35 uptake mediated by OATP8 and the export mediated by MRP2. Transfected MDCK cells were pre-incubated with different concentrations of CDNB at room temperature for 20 min before measurement of the transcellular [³H]BSP transport. As shown in

Fig. 9A, the intracellular accumulation and the transcellular transport of [³H]BSP in MDCK-OATP8 cells was not inhibited by CDNB up to a concentration of 50 μ M. However, CDNB exerted a completely different effect on MDCK-MRP2/OATP8 cells. The intracellular accumulation of [³H]BSP was enhanced, whereas the transcellular transport of [³H]BSP was markedly inhibited by the pre-incubation with CDNB (Fig. 9B). These results indicate that CDNB does not affect the OATP8-mediated uptake, but inhibits the MRP2-mediated export of [³H]BSP after formation of dinitrophenylglutathione inside the cells.

We have recently reported that human serum albumin (HSA) potently inhibits OATP8-mediated uptake of [³H]BSP, probably by binding [³H]BSP with very high affinity (Cui et al. 2001). Thus, we studied here the effect of HSA on the transcellular transport of [³H]BSP across the MDCKII transfectants. In the presence of 5 μ M HSA the OATP8-mediated accumulation of [³H]BSP in MDCK-OATP8 cells was suppressed almost completely (Fig. 9C). Also in the MDCK-MRP2/OATP8 cells, the [³H]BSP accumulation was strongly inhibited by HSA (Fig. 9D). Consequently, the transcellular transport of [³H]BSP was also diminished in the MDCK-MRP2/OATP8 cells (Fig. 9D).

OATP8-mediated uptake of [³H]E₂17bG into OATP8-transfected HEK293 cells can be inhibited by the antibiotics rifampicin and rifamycin SV (Cui et al. 2001). Fig. 9E-H demonstrate that both antibiotics interfere strongly with both OATP8 and MRP2. Consistent with our earlier studies (Cui et al. 2001), both antibiotics inhibited the intracellular accumulation of [³H]BSP in the MDCK-OATP8 cells (Fig. 9E and G). In the MDCK-MRP2/OATP8 cells however, the intracellular accumulation of [³H]BSP was only suppressed to about 50% of control, whereas the transcellular transport of [³H]BSP was inhibited more strongly by rifampicin and rifamycin SV (Fig. 9G and H). These results suggest that both compounds inhibit MRP2-mediated export in addition. This is confirmed by transport studies using membrane vesicles from HEK-MRP2 cells. Rifampicin at a concentration of 50 μ M inhibited [³H]LTC₄ transport into HEK-

MRP2 membrane vesicles to 50% of control, and 50 μM rifampicin SV inhibited LTC₄ transport into HEK-MRP2 membrane vesicles to 38% of control.

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Example 7: Transcellular Transport of Rifampicin

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Because rifampicin strongly inhibits both human OATP8 and MRP2, we were interested in whether the transcellular transport of rifampicin itself can be measured using our double-transfectants. We took the advantage of the intensive absorption of rifampicin at 475 nm to determine its concentration. A rifampicin concentration of as low as 1 μM could be measured by this method. As shown in Fig. 10, a significantly higher transcellular transport of rifampicin could be observed with MDCK-MRP2/OATP8 cells in comparison to the other three MDCKII transfectant cell lines. A rifampicin concentration of about 50 μM in these experiments was the lowest one that we could use to obtain rifampicin transport into the apical compartments in an amount detectable by the photometric method.

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Example 8: Additional double-transfected Cell Lines

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In addition to the above described double-transfected cell line MDCK-MRP2/OATP8 two further double transfected cell lines have been generated in the same manner: MDCK-OAT1/MRP2 and MDCK-OATP2/MRP2. The combination of the basolateral organic anion transporter OAT1 (gene symbol: SLC22A6; Hosoyomada et al, 1999) and the apical export pump MRP2 represents a model system for studies on renal transport processes. The combination of the basolateral organic anion transporter OATP2 (Abe et al., 1999; Hsiang et al, 1999; König et al., 2000a) and the apical export pump MRP2 represents a model system for studies on hepatic transport processes. The correct localisation of the recombinant transport proteins in the double-transfected cell lines was demonstrated by

immunofluorescence confocal laser scanning microscopy (Fig. 11).

Example 9: Transcellular Transport of para-Aminohippurate

The transport activity of the double-transfected cell line MDCK-OAT1/MRP2 was studied by the use of the widely used model compound para-aminohippurate (PAH). PAH has been used for studies on renal secretion of organic anions. In the renal proximal tubule epithelia, PAH is taken up from the blood at the basolateral membrane by OAT1 (Hosoyamada et al., 1999). Subsequently, PAH is secreted ATP-dependently by MRP2 (Leier et al, 1999) into the urine. As demonstrated in Fig. 12, the rate of transcellular transport of PAH is significantly higher in the double-transfected cell line MDCK-OAT1/MRP2 than in the control-transfected cell line MDCK-Co and in the single-transfected cell line MDCK-OAT1.

Example 10: Transcellular Transport of BSP

The transport activity of the double-transfected cell line MDCK-OATP2/MRP2 was studied by the use of the model compound BSP. As demonstrated in Fig. 13, the rate of transcellular transport of BSP is significantly higher in the double-transfected cell line MDCK-OATP2/MRP2 than in the control-transfected cell line MDCK-Co and in the single-transfected cell line MDCK-OATP2.

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Claims

- 5 1. A double-transfected cell line containing (a) a DNA sequence encoding an uptake transporter for organic anions operatively linked with a promoter and (b) a DNA sequence encoding an export pump for organic anions or anionic conjugates operatively linked with a promoter.
- 10 2. The cell line of claim 1 which is a canine or human cell line and the DNA sequences of (a) and/or (b) are human.
3. The cell line of claim 1 or 2 which is a kidney cell line.
- 15 4. The cell line of any one of claims 1 to 3 wherein the uptake transporter for organic anions is a member of the subgroup 21A or 22A of the solute carrier (SLC) superfamily.
- 20 5. The cell line of claim 4, wherein the uptake transporter for organic anions is OAT1 (SLC22A6), OATP2 (SLC21A6), OATP8 (SLC21A8) or OATP-B (SLC21A9).
- 25 6. The cell line of any one of claims 1 to 5 wherein the export pump for organic anions or anionic conjugates is a member of the MDR (ABCB) subgroup or the MRP (ABCC) subgroup of the ABC superfamily.
- 30 7. The cell line of claim 6, wherein the export pump for organic anions or anionic conjugates is the bile salt export pump BSEP (ABCB11) or the multidrug resistance protein 2 (MRP2; ABCC2).
- 35 8. The cell line of any one of claims 1 to 7 wherein the DNA sequence encoding an uptake transporter for organic anions and/or the DNA sequence encoding an export pump for organic anions or anionic conjugates are operatively linked with a promoter allowing high expression.

9. Use of a cell line according to any one of claims 1 to 8 for the identification of a transport substrate or a transport inhibitor.
- 5 10. Use according to claim 9 wherein the transport inhibitor is a drug candidate.
- 10 11. Use according to claim 9 or 10 wherein the identification of a transport substrate or a transport inhibitor is carried out as high throughput screening.

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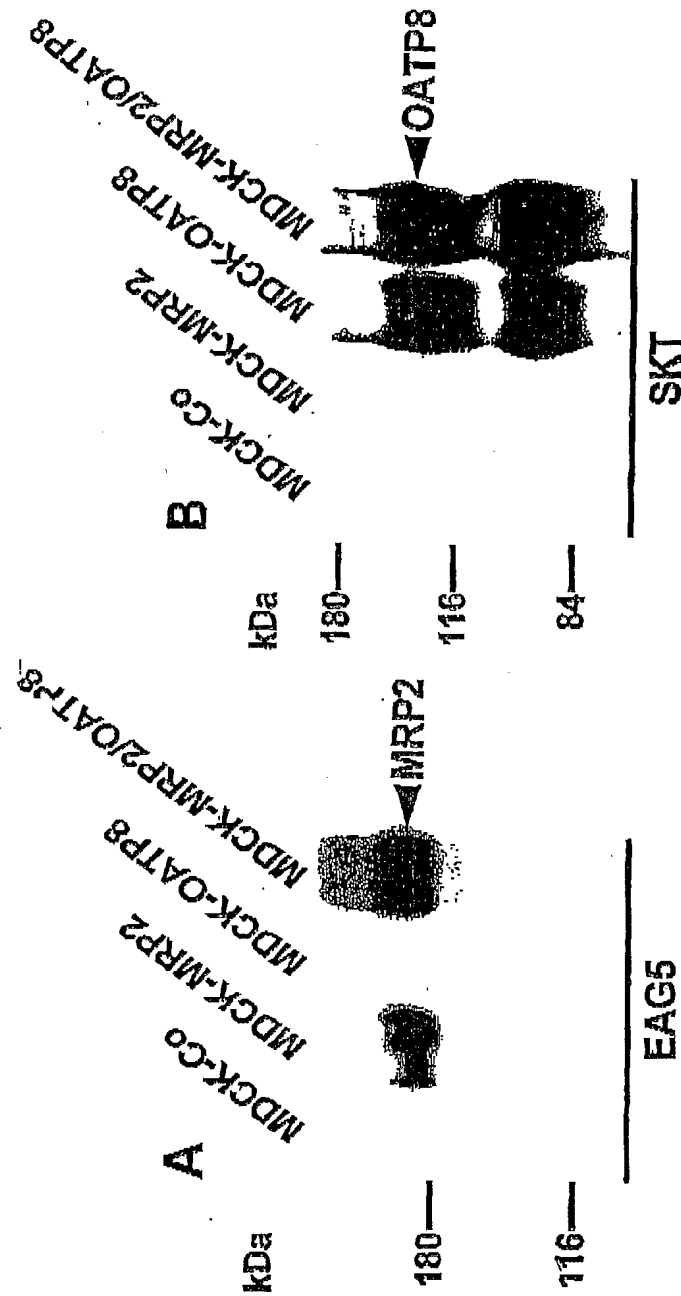


Fig. 1.

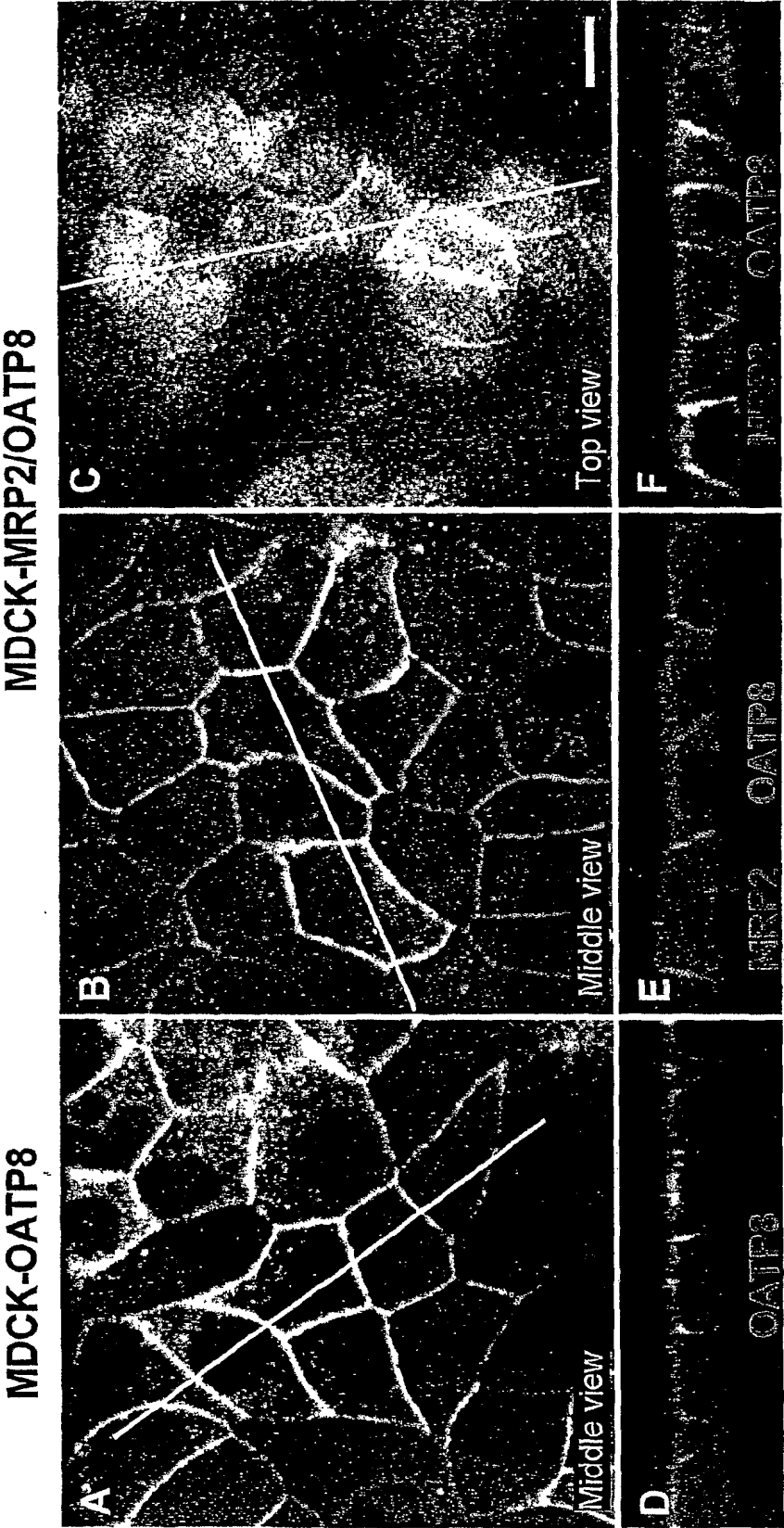


Fig. 2.

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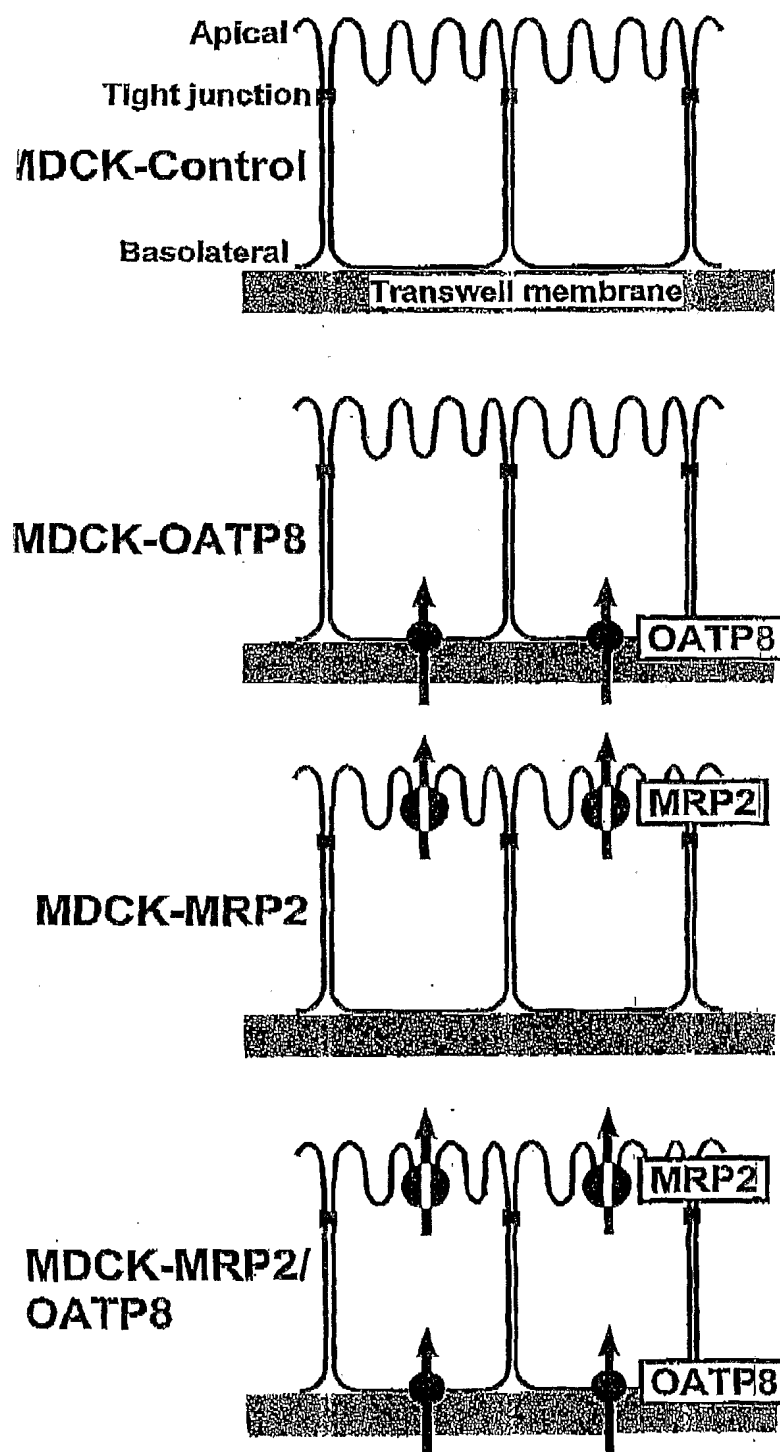


Fig. 3

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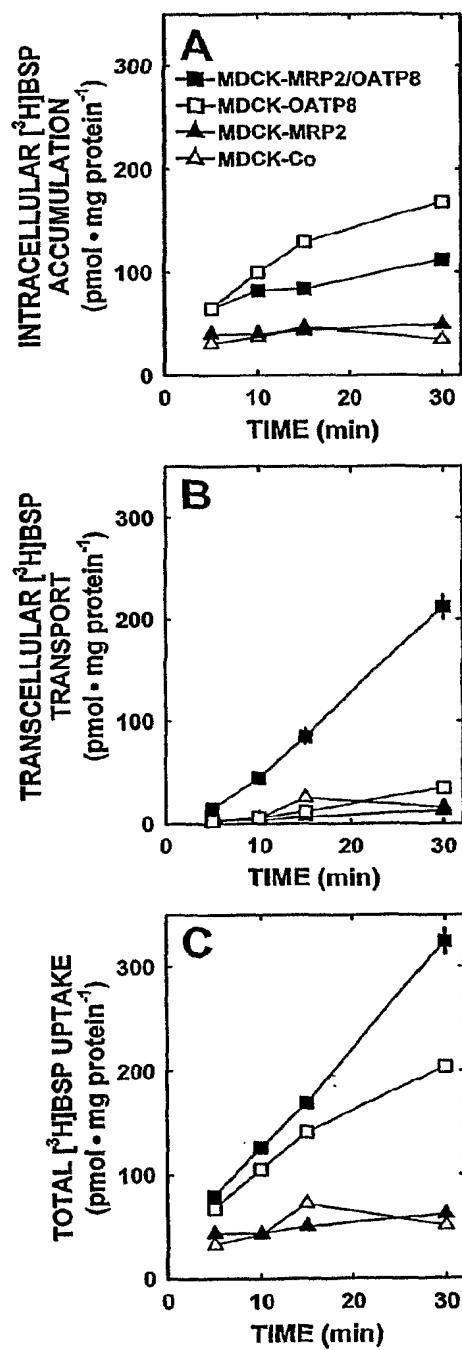


Fig. 4.

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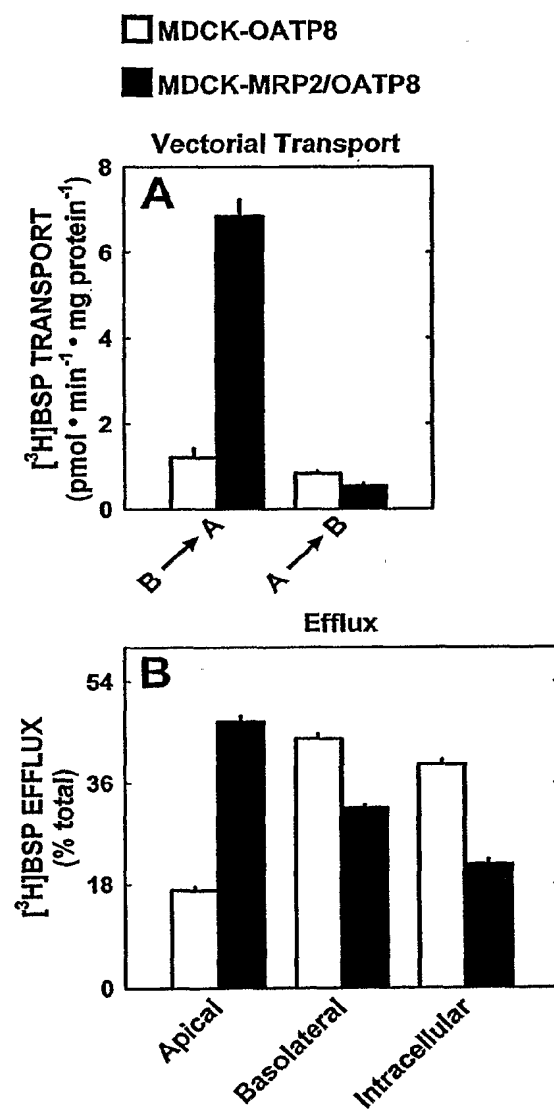


Fig. 5.

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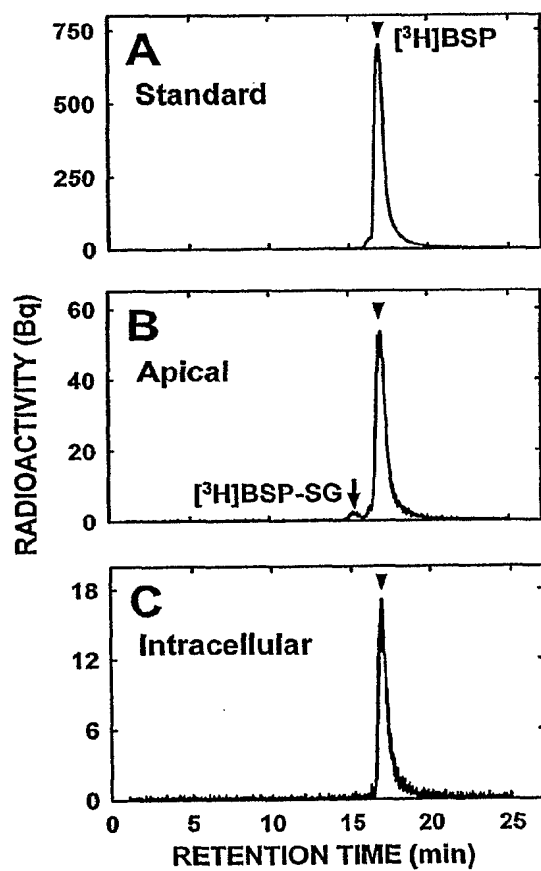


Fig. 6.

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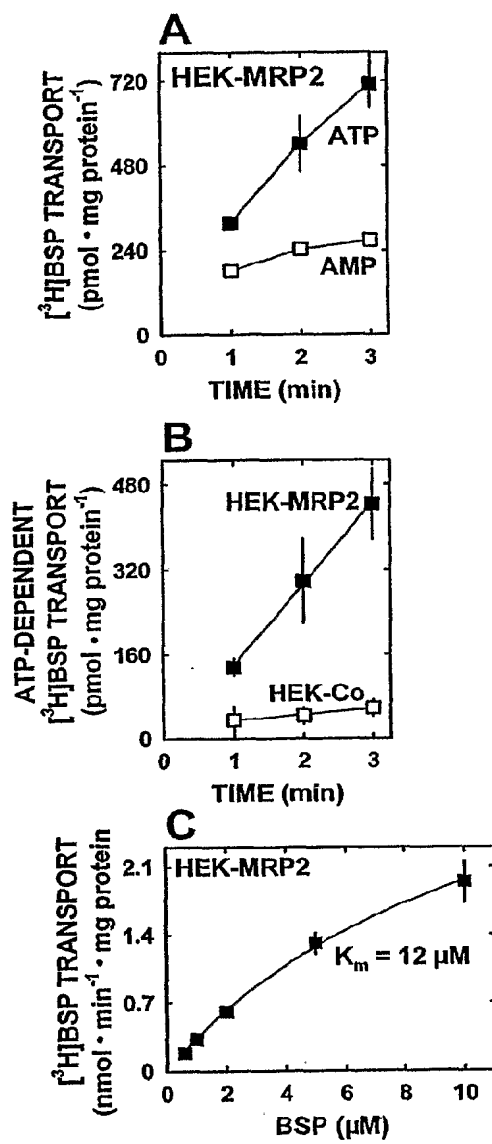


Fig. 7.

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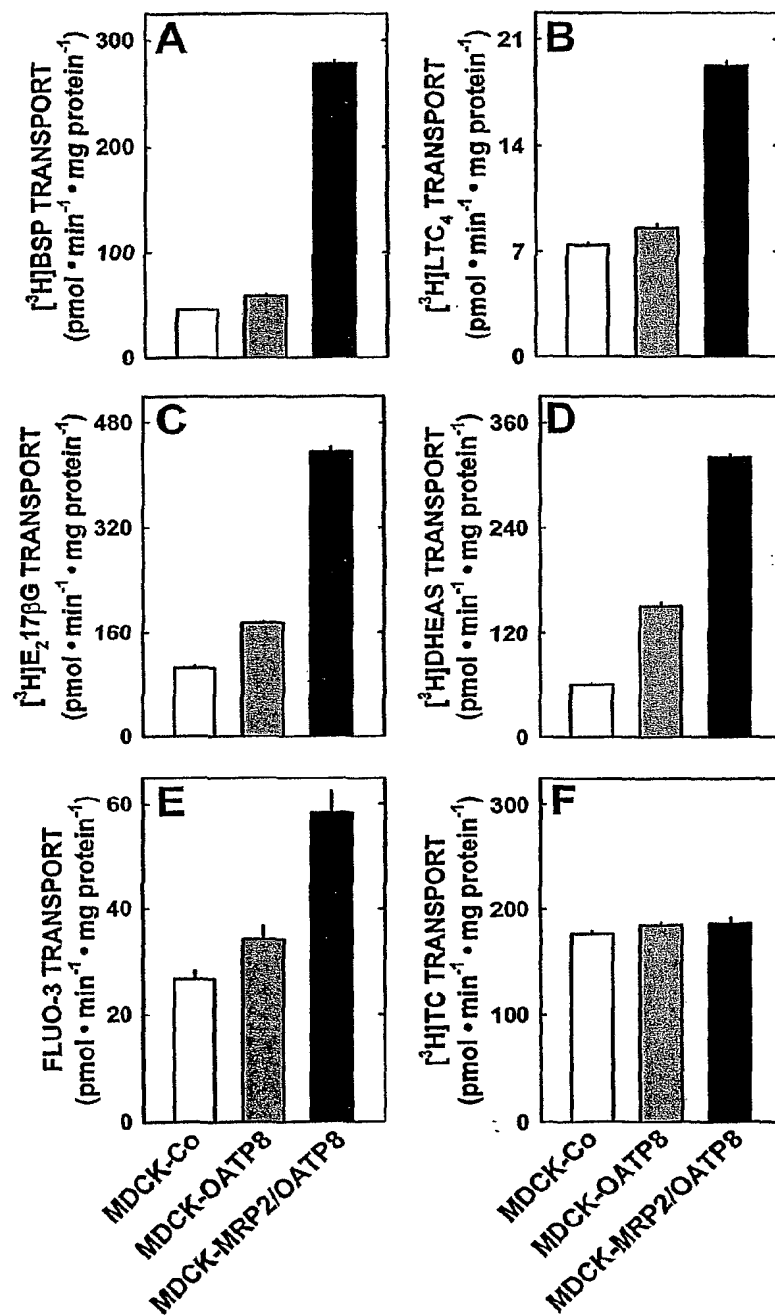
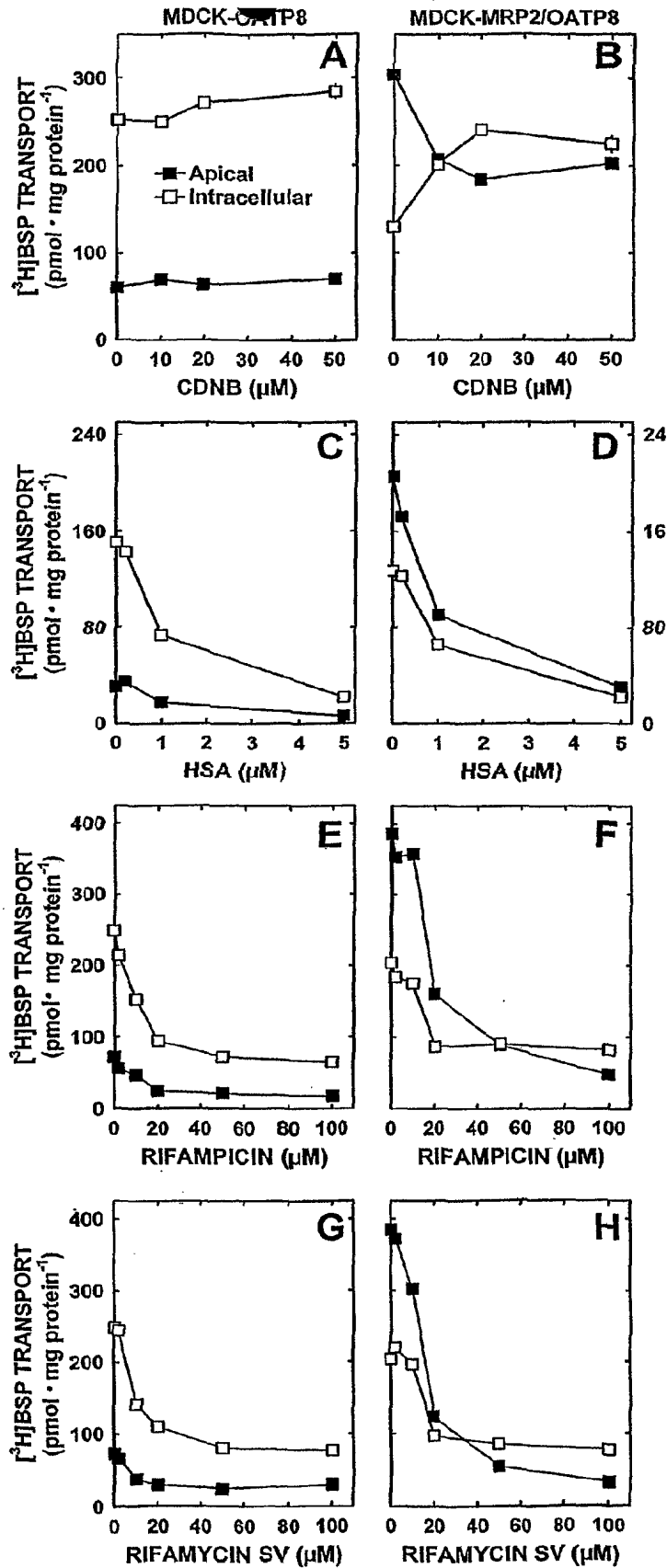


Fig. 8.

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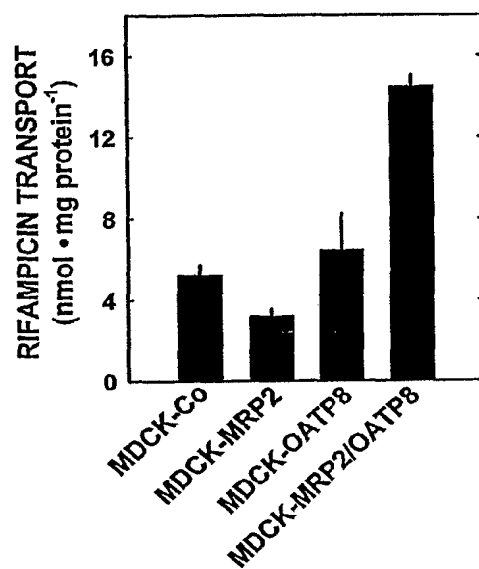


Fig. 10.

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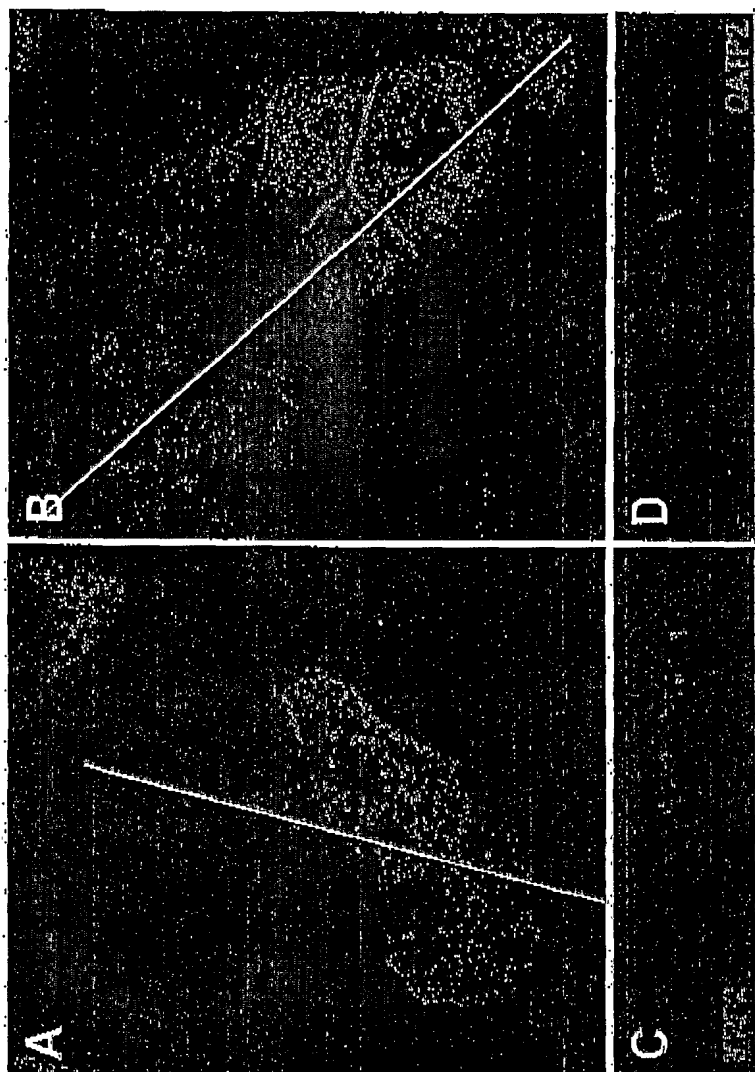


Fig. 11.

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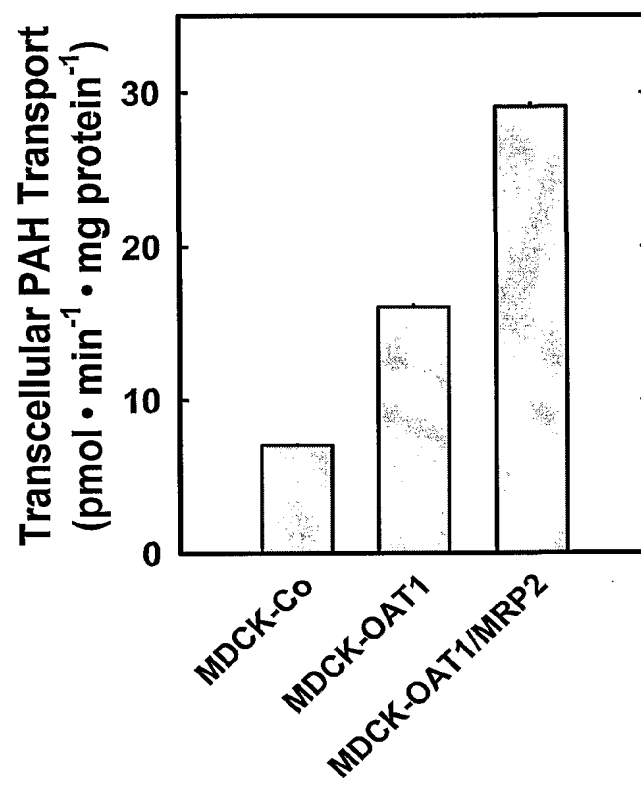


Fig. 12.

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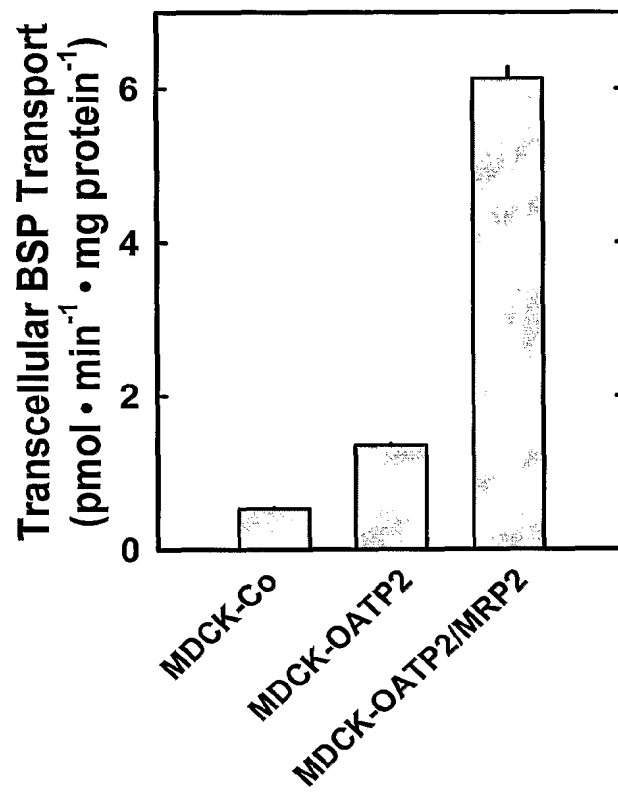


Fig. 13.

INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 02/06175

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 7 C12N5/10 G01N33/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

MEDLINE, BIOSIS, EPO-Internal, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>CUI YUNHAI ET AL: "Hepatic uptake of bilirubin and its conjugates by the human organic anion transporter SLC21A6." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 276, no. 13, 30 March 2001 (2001-03-30), pages 9626-9630, XP002209309 ISSN: 0021-9258 the whole document</p> <p style="text-align: center;">--- -/--</p>	1-11



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

° Special categories of cited documents:

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O document referring to an oral disclosure, use, exhibition or other means

P document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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G document member of the same patent family

Date of the actual completion of the international search

9 August 2002

Date of mailing of the international search report

23/08/2002

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Herrmann, K

INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 02/06175

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>HSIANG BONNIE ET AL: "A novel human hepatic organic anion transporting polypeptide (OATP2): Identification of a liver-specific human organic anion transporting polypeptide and identification of rat and human hydroxymethylglutaryl-CoA reductase inhibitor transporters." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 274, no. 52, 24 December 1999 (1999-12-24), pages 37161-37168, XP002209310 ISSN: 0021-9258 the whole document</p>	1-11
A	<p>CUI YUNHAI ET AL: "Drug resistance and ATP-dependent conjugate transport mediated by the apical multidrug resistance protein, MRP2, permanently expressed in human and canine cells." MOLECULAR PHARMACOLOGY, vol. 55, no. 5, May 1999 (1999-05), pages 929-937, XP002209311 ISSN: 0026-895X the whole document</p>	1-11
A	<p>CANTZ TOBIAS ET AL: "MRP2, a human conjugate export pump, is present and transports fluo 3 into apical vacuoles of Hep G2 cells." AMERICAN JOURNAL OF PHYSIOLOGY, vol. 278, no. 4 part 1, April 2000 (2000-04), pages G522-G531, XP001095647 ISSN: 0002-9513 the whole document</p>	1-11
P,X	<p>CUI YUNHAI ET AL: "Vectorial transport by double-transfected cells expressing the human uptake transporter SLC21A8 and the apical export pump ABCC2." MOLECULAR PHARMACOLOGY, vol. 60, no. 5, November 2001 (2001-11), pages 934-943, XP001098543 ISSN: 0026-895X the whole document</p>	1-11